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ORIGINAL ARTICLE

In vitro multiplication of *Dianthus carthusianorum* calamine ecotype with the aim to revegetate and stabilize polluted wastes

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Abstract Abandoned metalliferous wastes can be spontaneously colonized by specialized species or ecotypes, and therefore representatives from such populations might be exploited in phytoremediation. Thus, this study was focused on determining the conditions for culture initiation and elaborating the propagation protocol of wild calamine ecotype of *Dianthus carthusianorum*. The proper propagation medium proved modified MS enriched with 1.0 mg/L 2iP and 0.2 mg/L IAA. The massive majority (93%) of microplantlets were successfully transferred to *ex vitro* conditions. Micropropagated calamine ecotype of *D. carthusianorum* has proved to be useful for phytoremediation application. The obtained plants experimentally cultivated on post-flotation wastes generated during the process of zinc-lead ore enrichment were monitored, and compared with specimens of the population obtained as a result of seed sowing. Plants propagated through tissue culture grew better, developed faster and more abundantly bloomed in comparison with the generatively propagated control material. This is one of the few reports concerning the possibility of using in vitro technique for effective production of plant material ready to be used in chemically degraded area.

Keywords Carthusian pink · Heavy metals · In vitro · Metallophytes · Phytoremediation · Waste heap

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Abbreviations

2iP	2-isopentenyladenine
BAP	6-benzylaminopurine
IAA	Indole-3-acetic acid
NAA	1-naphtaleneacetic acid
MES	2-N-morpholino-ethanesulfonic acid
PVP	Polyvinylpyrrolidone

Introduction

Metal mining and smelting activities have a deleterious effect on the environment due to the production of huge waste amounts that are often a major source of pollution and have an undesirable aesthetic impact on the local landscape. Moreover, such waste deposits are characterized by low concentration of nutrients and highly elevated levels of certain heavy metals, water deficiency, and frequently unfavourable pH in the substratum as well as high insolation and strong winds (Przedpełska and Wierzbicka 2007; Muszyńska et al. 2013; Wójcik et al. 2014). As a result, in those harsh conditions only the specialized plant species are able to survive due to the existence of adaptive mechanisms. Carthusian pink (*Dianthus carthusianorum* L.) is a polymorphic species, belonging to Caryophyllaceae family, which has developed different ecotypes in Europe. It can grow well in both uncontaminated and metal-contaminated sites on dry and the sun exposed edges of fields, rocky slopes and fallow land. It is also one of the dominant plant species on more than 100 years old calamine waste heaps in Olkusz district (southern Poland) rich in lead and zinc ions (Szarek-Łukaszewska 2009; Nowak et al. 2011). Studies on this species have shown that the plants of metalliferous ecotype are significantly different in their morphology, physiology and genetic features from specimens

representing the same species occurring on unpolluted soils (Wierzbicka and Rostański 2002; Baranowska-Morek and Wierzbicka 2004; Wójcik and Tukiendorf 2014; Wójcik et al. 2014). The described morphologic differences mainly point out to the good adaptation to xerothermic conditions. Moreover, specimens from the calamine population exhibit a high level of tolerance to lead, zinc and cadmium in comparison with specimens from non-metalliferous populations (Ciarkowska and Hanus-Fajerska 2008; Wójcik and Tukiendorf 2014; Wójcik et al. 2015). Thus, calamine population, like other metal-tolerant plant taxa, should be suitable for phytoremediation, i.e. eco-friendly and cost-effective technology that uses plants to remove contaminants from the environment or to decrease their toxicity (Ali et al. 2013). One of the several known phytoremediation techniques is phytostabilization that involves plants for the formation of vegetative cover where at the same time sequestration (binding and sorption) processes immobilize metals within the rhizosphere. Consequently, the metal bioavailability is reduced and the exposure of other trophic levels of the ecosystem is restricted. The green dense vegetative cover prevents eolian dispersion of contaminated dusts while also helping to decrease water erosion and leaching. Therefore, phytostabilization is applied for the long-term stabilization of the metalliferous mine wastes (Sheoran et al. 2013).

Taking into account the possibility of practical application of tolerant ecotypes, it is essential to elaborate efficient methods of their reproduction, aiming at growing them on such chemically degraded soils. Such specific plant material is meant to be exploited in innovative in situ method of contaminated soils remediation. For the reasons mentioned above, the aim of the present research was to determine conditions for in vitro culture initiation, and to elaborate the propagation protocol of *Dianthus carthusianorum* ecotype from calamine industrial area. It was an important initial step in the long-term strategy aiming to revegetate highly degraded stands in the region where those *D. carthusianorum* population had previously evolved.

Materials and Methods

Plant material culture condition and experimental schedule

Donor material to initiate in vitro experiments have been seed samples of *Dianthus carthusianorum* L. (Caryophyllaceae Juss.) collected from the population which spontaneously appears on an old waste heap obtained after mining and processing of Zn-Pb ores in Bolesław near Olkusz (the Silesia-Cracow Upland, Poland). The seeds were immersed in 70% (v/v) ethanol for 1 min and surface decontaminated with 0.1% mercuric chloride for 5 min. After three washes with sterile distilled water, they were placed onto MS medium without plant growth regulators (Murashige and Skoog 1962). Shoot tips of aseptically obtained seedlings were used as primary explants to establish proliferating shoot culture. Excised seedling shoots bearing an apical meristem were placed onto (1) modified MS (Murashige and Skoog 1962) supplemented with 20 g/L sucrose, 0.65 g/L calcium gluconate, 0.5 g/L polyvinylpyrrolidone (PVP), 0.5 g/L 2-N-morpholinoethanesulfonic acid (MES), or (2) modified Woody Plant Medium (Lloyd and McCown 1980) consisted of WPM salts, MS vitamins plus 0.3 g/L activated charcoal. The media were solidified with 0.75% Difco Bacto agar, and their pH was adjusted to 5.8 before autoclaving (121 °C for 20 min).

The following media based on MS modifications are further referred to as MS, and the media based on WPM salts are referred to as WPM. The composition of tested plants growth regulators added to either MS or WPM medium are presented in Table 1. Cultures were maintained in air-conditioned chamber at 24 °C day/20 °C night under 16 h light photoperiod regime with irradiance 80 µm m^{-2} s^{-1} PAR. As a light source cool white fluorescent lamps were applied (Philips TL 33).

The cultures were checked macroscopically every 7 days, and micropropagation coefficient (MC) was calculated using the following formula:

MC = number of induced adventitious shoots/total number of explaints

Shoots (as well as roots if developed) were not only measured, but fresh weight was also taken at the end of the experiment, that is after 12 weeks of culture. Respective samples were then oven dried in 105 °C for 24 h to weigh their dry matter.

Table 1 The tested combination of plants growth regulators added to either modified MS or WPM medium and culture medium code depending on their composition

Basal medium	Plant growth regulators	Culture medium code
MS	1.0 mg/L BAP + 0.2/L NAA	*D1*
	1.0 mg/L 2iP + 0.2 mg/L NAA	*D2*
	1.0 mg/L BAP + 0.2 mg/L IAA	*D3*
	1.0 mg/L 2iP + 0.2 mg/L IAA	*D4*
WPM	2.5 mg/L 2iP + 1.0 mg/L IAA	*D5*
	1.0 mg/L 2iP + 0.2 mg/L IAA	*D6*

In the course of micropropagation experiment, the Erlenmeyer flask of 100 cm^3 capacity filled with 20 cm^3 of respective media was used and five shoot explants were put in the single flask onto freshly prepared medium. In total 50 explants in every treatment were evaluated (ten flasks per treatment). The whole experimental set was repeated three times. The data were subjected to ANOVA analysis (STATISTICA 10.0, StatSoft, Tulsa, OK, USA) and a post-hoc Fisher's test was performed to determine differences between treatment at $\alpha = 0.05$.

The selection of the best propagation medium

In order to find the optimal propagation medium, each value of examined characteristic was given a rank according to the intensification of examined characteristics. Numbering was started at 1. The lowest value (1) was assigned to the statistically defined homogeneous group with the lowest intensity of analysed characteristic, while the highest—to the group with the highest intensity. The statistically homogeneous overlapping groups got the average points of both groups. Groups in which there was no occurrence of the trait received no points (0). The following characteristics were evaluated: micropropagation coefficient, shoot length, root length and their number per explant as well as shoot and root fresh and dry matter. The best micropropagation medium got the highest total value calculated on the basis of summation of all the scores defined for individual traits.

The acclimatization step

More than 60 of spontaneously rooted plantlets (R_0) representing D4 treatment were transplanted to ceramic pots 90 mm in diameter with autoclaved mixture of perlite and horticultural soil (1:1 v/v). During the first 2 weeks, plantlets were protected with transparent containers in order to provide optimum humidity (relative humidity 70%), and afterwards they were transferred to the isolated bottom-heated (18–20 °C) section in the greenhouse. The percentage of survived specimens was calculated after 8 weeks, and at that time they were transplanted to bigger pots (100 mm of diameter) containing a mixture of perlite, horticultural soil and post-flotation wastes obtained in the process of zinc-lead ores enrichment (1:1:3 v/v).

The comparison of specimens obtained by vegetative or generative propagation

To evaluate the usefulness of in vitro techniques for studied production of *D. carthusianorum* ecotype, growth and development of specimens obtained under in vitro conditions on D4 medium were compared in greenhouse conditions with the plants obtained conventionally via seeds sowing. The initial plant material for generative propagation were seed samples collected from the same calamine population as those used for in vitro culture initiation. The comparison was conducted during the second year of cultivation when both groups of plants (R_0—obtained in vitro/from seed sowing) were in the same developmental stage, and grew on substratum supplemented with post-flotation wastes (the same as previously used for acclimatization of R_0 plants). Twenty plants representing both propagation types were closely observed during their growth on field plot located in front of the greenhouse. The rate of growth, plant diameter and number of shoots were estimated at least three times each month, and the measurements began in April. Additionally, the percentage of flowering specimens, inflorescence number per specimen, their height as well as flower number per inflorescence and flower diameter were evaluated.

Results

In vitro culture initiation and shoot multiplication

In order to initiate in vitro culture, some sterilizing agents were primarily tested (data not shown). The most effective surface decontamination of *D. carthusianorum* seeds was achieved using 0.1% solution of $HgCl_2$ for 5 min. Those seeds germinated on average at 95.5% and the greatest number of properly shaped seedlings that could easily develop to aseptic plantlets were obtained in such a case. The morphogenetic potential of obtained cultures was proved to be a variable depending on particular medium treatment. Despite the applied medium, new shoots were formed by axillary branching and no callus proliferation was observed. As expected, cultures proliferated less vigorously on applied modification of WPM medium than MS (Table 2; Fig. 1a–f), and stronger elongation of shoots, the length of which exceeded 40- on D6 and 55 millimetres on D5 medium was noticed. Moreover, WPM medium supplemented with 1.0 mg/L 2iP and 0.2 mg/L IAA (medium D6, Fig. 1a) did not stimulate spontaneous regeneration of adventitious roots, while at the same medium but supplemented with 2.5 mg/L 2iP and 1.0 mg/L IAA (medium D5, Fig. 1b) abundant spontaneous rhizogenesis was observed, and the number of regenerated roots reached the highest value, i.e. 14 roots per shoot clump. It can be quite an interesting result when we are interested in rooting of elongated shoots under *ex vitro* conditions. However, the roots regenerated on D5 were much shorter than in the case of cultures maintained on MS medium enriched with the same growth regulators, but at lower concentrations (medium D4, Fig. 1c). The greatest shoot multiplication coefficient (MC = 13) was ascertained on MS supplemented with

Table 2 Effect of the different media composition on *D. carthusianorum* growth parameters after 12 weeks of in vitro cultivation (means ± SE)

Culture medium	Multiplication coefficient	Shoots length (mm)	Shoot fresh weight (g)	Shoot dry weight (g)	Roots number/explants (pcs)	Roots length (mm)	Root fresh weight (g)	Root dry weight (g)
D1	1.67^{cd}*	33.86 ± 3.43^{b}	0.400 ± 0.21^{b}	0.0294 ± 0.002^{ac}	2.67 ± 1.65^{bc}	29.88 ± 3.72^{a}	0.017 ± 0.003^{ab}	0.0014 ± 0.002^{a}
D2	3.89^{b}	25.82 ± 6.81^{d}	0.633 ± 0.10^{a}	0.0472 ± 0.002^{a}	1.00 ± 0.83^{c}	15.89 ± 4.21^{b}	0.007 ± 0.007^{b}	0.0010 ± 0.002^{a}
D3	13.13^{a}	21.45 ± 5.16^{d}	0.668 ± 0.11^{a}	0.0382 ± 0.001^{ab}	0.00	0.00	0.000	0.0000
D4	2.33^{c}	35.10 ± 7.54^{bc}	0.665 ± 0.21^{a}	0.0452 ± 0.002^{a}	3.91 ± 1.31^{b}	32.68 ± 1.71^{a}	0.033 ± 0.010^{a}	0.0019 ± 0.002^{a}
D5	1.00^{d}	57.93 ± 2.28^{a}	0.113 ± 0.02^{c}	0.0130 ± 0.005^{c}	14.20 ± 3.10^{a}	8.71 ± 3.82^{c}	0.015 ± 0.008^{ab}	0.0016 ± 0.001^{a}
D6	1.00^{d}	43.51 ± 5.71^{b}	0.205 ± 0.05^{bc}	0.0174 ± 0.005^{c}	0.00	0.00	0.000	0.0000

*Values are means of three replicates, means indicated by the same letter within the columns do not significantly differ at $\alpha = 0.05$ according to Fisher's test

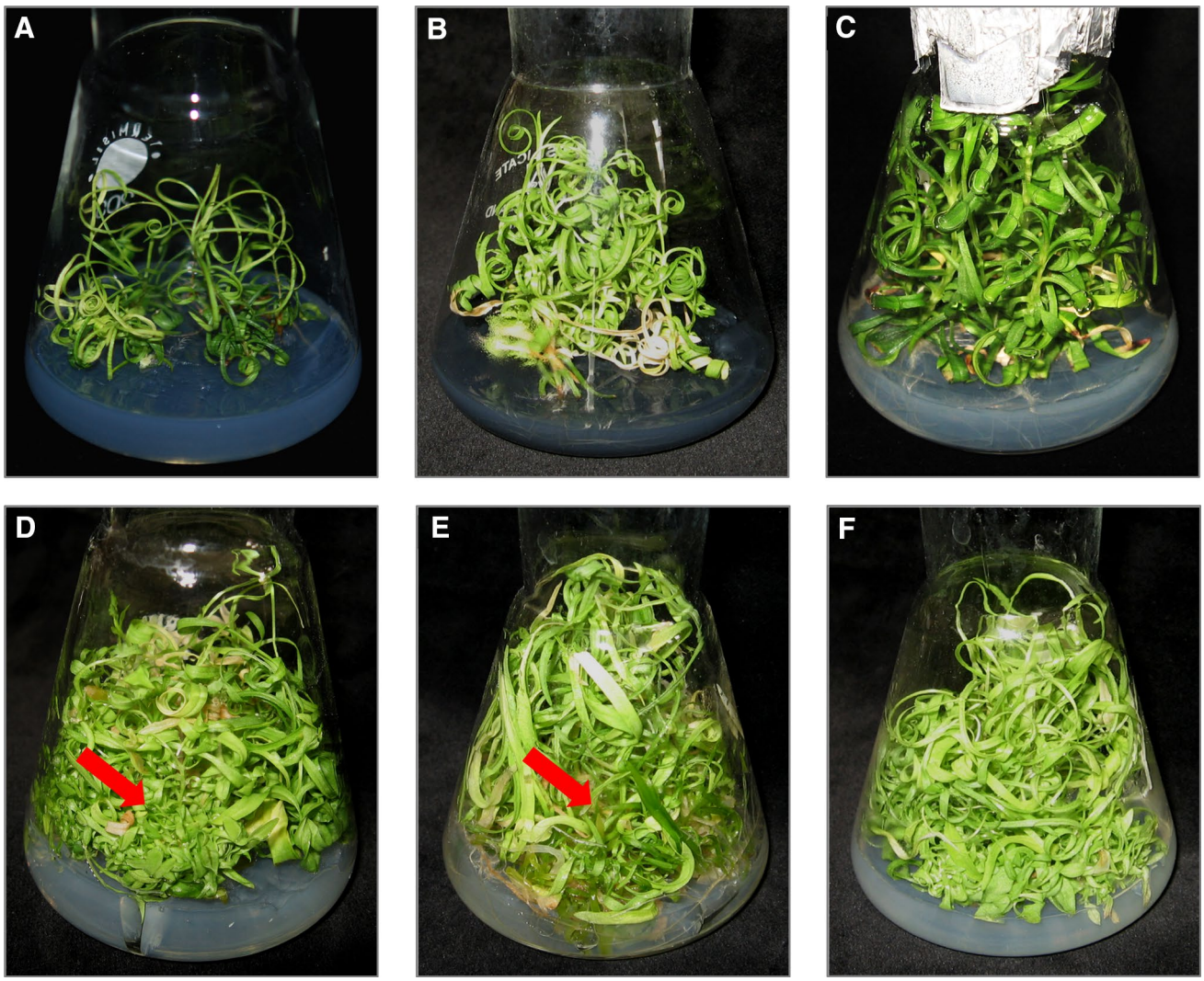

Fig. 1 Micropropagation of *D. carthusianorum* calamine ecotype on media enriched with different combination of cytokinins and auxins (12 week of cultivation). **a** Propagation on WPM medium enriched with 1.0 mg/L 2iP and 0.2 mg/L IAA. **b** Propagation on WPM medium enriched with 2.5 mg/L 2iP and 1.0 mg/L IAA. **c** Shoots regeneration on MS medium enriched with 1.0 mg/L 2iP and 0.2 mg/L IAA (considered as optimal for clonal propagation). **d, e** Thick, vitreous and curly shoots regenerated on MS medium enriched with 1.0 mg/L BAP and 0.2 mg/L IAA. **f** Culture growth on MS medium enriched with 1.0 mg/L 2iP and 0.2 mg/L NAA

1.0 mg/L BAP and 0.2 mg/L IAA (medium D3). Nevertheless, in such a case regenerated shoots were relatively short (about 21 mm long), thick and sometimes vitreous (Fig. 1d–e). In comparison with the culture maintained on the medium D3, statistically significant reduction in the number of regenerated shoots from a single explant (MC = 2) was noted on cultures treated with 1.0 mg/L 2iP and 0.2 mg/L IAA (medium D4). Despite the differences in the number of shoots and their length on D3, D4 as well as on D2 medium (Fig. 1f), fresh and dry matter content was proved to be similar because it amounted approximately to 0.65 g for fresh matter and ranged from 0.038 to 0.047 g in case of dry biomass (Table 2).

The selection of the best propagation medium

The most convenient medium for clonal propagation of studied *D. carthusianorum* ecotype was chosen on the basis of the highest total value which was obtained after the summation of the all scores defined for individual examined characteristics (Fig. 2). Although the cultures growing on D4 medium produced fewer shoots per explant than on MS media with other combinations of plants growth regulator (Table 2), the highest total value resulting from the greatest development of microcuttings was noted in this treatment. Thus, MS medium enriched with 1.0 mg/L and 0.2 mg/L IAA was chosen for micropropagation of Carthusian pink.

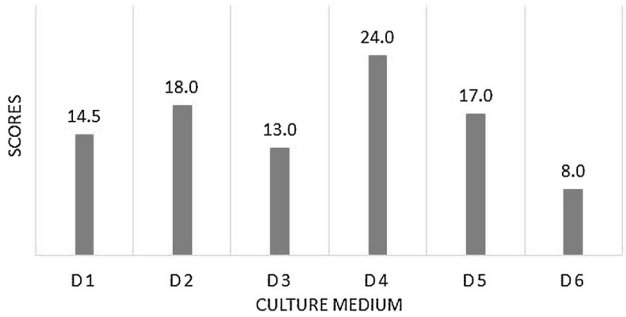

Fig. 2 Total scores defined for individual examined traits during optimal medium selection for micropropagation of *D. carthusianorum*

The acclimatization of obtained microplants

In view of the best growth and the most proper shape of shoots regenerated on D4 medium, only microcuttings obtained in this treatment were transferred to a sterile mixture of perlite and horticultural soil in 1:1 ratio. Due to the abundant spontaneous rhizogenesis, the proper rooting phase could be omitted in such treatment. More than 60 (65) rooted microcuttings were transplanted to *ex vitro* condition. Despite the protection with transparent containers, the strong turgor loss of shoot was observed during the first week of acclimatization (Fig. 3a). Nevertheless, plants survival after 2 months was high and reached 93% (61 of survived plants). Then, they were transplanted to bigger containers containing wastes material coming from post-flotation settling pond (Fig. 3b). In this step of experiment 100% survival rate was obtained, and no negative effects of wastes on *D. carthusianorum* growth and development were observed.

Vegetative versus generative propagation of *D. carthusianorum* calamine ecotype

During the field cultivation on substratum enriched with post-flotation wastes, statistically significant differences were ascertained between specimens propagated vegetatively with the use of in vitro technique and those obtained as a result of generative propagation (Table 3;

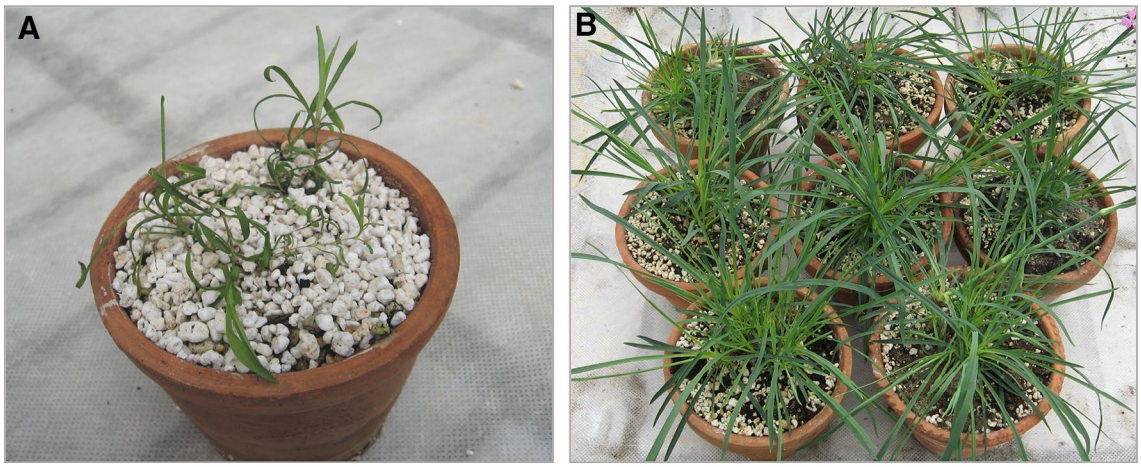

Fig. 3 Specimens of *D. carthusianorum* calamine ecotype transferred to *ex vitro* conditions: **a** Small plants during the first week of acclimatization. **b** Plants transplanted to post-flotation wastes after 10 weeks of *ex vitro* growth

Table 3 The growth parameters comparison of *D. carthusianorum* specimens obtained by micropropagation (vegetative) and by seed sowing in greenhouse condition (generative) during their cultivation on post-flotation wastes in the field (means ± SE)

Feature		Plant diameter (mm)	Shoots number (pcs)	Flowering specimens (%)	Inflorescence number/specimen (pcs)	Inflorescence height (mm)	Flower number/inflorescence (pcs)	Flower diameter (mm)
Measurement								
I*	Vegetative	77.91 ± 4.17^{a}**	6.00 ± 0.93^{a}	50.00	3.20 ± 0.64	152.71 ± 10.21	4.30 ± 0.33	Buds
	Generative	59.82 ± 5.31^{b}	4.40 ± 0.84^{b}	–	–	–	–	–
II	Vegetative	92.90 ± 5.08^{a}	12.90 ± 2.21^{a}	90.00^{a}	5.87 ± 1.16^{a}	327.51 ± 15.22^{a}	5.12 ± 0.78^{a}	20.92 ± 1.57
	Generative	81.39 ± 3.43^{b}	7.33 ± 1.88^{b}	60.00^{b}	3.16 ± 0.75^{b}	233.68 ± 11.37^{b}	3.00 ± 0.81^{b}	Buds
III	Vegetative	109.83 ± 4.94^{a}	21.15 ± 2.24^{a}	100.00^{a}	6.11 ± 1.36^{a}	348.12 ± 26.44^{a}	4.37 ± 0.92^{a}	20.29 ± 1.22^{a}
	Generative	91.45 ± 4.01^{b}	15.36 ± 2.37^{b}	90.00^{b}	5.87 ± 1.72^{a}	324.30 ± 19.32^{a}	3.77 ± 0.67^{b}	20.15 ± 0.82^{a}

*Subsequent term of measurements started in the second year of field cultivation (April) and performed at 4 week intervals

**Values are means of ten individuals, means indicated by the same letter within the terms of measurement do not significantly differ at $\alpha = 0.05$ according to Fisher's test

Fig. 4 Specimens of *D. carthusianorum* calamine ecotype obtained by generative and vegetative propagation growing on experimental field plot: **a** The comparison of plants growth propagated by seed sowing in greenhouse condition (*above*) and by micropropagation (*below*). **b** Flowering of plants propagated with the use of in vitro techniques (May 2013)

Fig. 4a–b). The first mentioned experimental population (R_0) grew and developed more vigorously, so that in April plant diameter reached almost 80 mm with the number of shoots equal to six. At the same time, the average value of respective parameter in specimens obtained from seed sowing was about 20% lower. In the course of further growth, statistically significant differences in respect to plant diameter as well as the shoot number unchanged. Furthermore, we noticed that plants obtained in vitro started to flower earlier (in April), they had better flower setting and higher inflorescences in comparison with the plants obtained as a result of generative propagation (seed sowing) (Fig. 4b). In specimens propagated vegetatively under in vitro conditions, the fully-developed flowers (with a diameter of 21 mm) appeared in May while the second group of plants just started blooming.

Discussion

Numerous characteristics of metalliferous wastes are extremely unfavourable for successful establishment of vegetation cover that could provide the necessary surface stability to prevent dust blow and leaching the contaminants into nearby watercourses (Tordoff et al. 2000; Szarek-Łukaszewska 2009; Muszyńska et al. 2013). Thus, enormous efforts should be undertaken to overcome the limited possibilities of plant introduction and survival on degraded areas. Novel sustainable strategy of waste stabilization and

reclamation bases on different metallophytes occurring on post-industrial terrains abandoned for many years (Nouri et al. 2011; Muszyńska et al. 2015; Pandey et al. 2015; Muszyńska and Hanus-Fajerska 2016). Metal-tolerant species are unique among vascular plants because of their adaptation to severe conditions as well as high ability to cope with elevated levels of heavy metals in soil or even in the soilless substrate such as waste-heaps. These features make them useful for establishment of a self-sustaining vegetative cover. Therefore, it is simply necessary to elaborate the very efficient methods of their production. Satisfactory multiplication of species with tolerance to high levels of toxic substances can be obtained by in vitro techniques. The development of effective micropropagation protocol is particularly valuable in the case of this unique flora whose representatives are described in varied ecological niches. A good example of this kind of scientific activity is multiplication of metal-tolerant species which grow on terrains with high metallic background (Doran 2009; Cristea et al. 2013; Jarda et al. 2014; Slazak et al. 2015), as the efficient regeneration protocol of *Thlaspi caerulescens*-perhaps the most famous hyperaccumulator of zinc and cadmium (Xu et al. 2008) or *Pteris vittata*-fern conducting phytovolatilization of arsenic (Zheng et al. 2008; Shukla and Khare 2014). Nevertheless, the investigations with the use of in vitro techniques for metallophyte propagation are still limited in comparison with well elaborated plant tissue culture protocols of numerous cultivated species (Bidwell et al. 2001; Jack et al. 2005; Hanus-Fajerska et al. 2009, 2012; Zhao et al. 2009; Wiszniewska et al. 2015). Therefore, the present study should be considered as an innovative approach to the protection of gene pool of this precious plant material. At the same time an ample supply of uniform material ready to be applied in phytoremediation schemes can easily be obtained by the use of in vitro techniques (Fig. 5).

The best growth of calamine *D. carthusianorum* culture was observed on modified MS medium. Similarly, MS was used in experimental work on *D. spiculifolius* (Cristea et al. 2013) or *D. giganteus* ssp. *banaticus* (Jarda et al. 2014) carried out in order to protect biodiversity. Another example could be *D. caryophyllus* that is one of the world's most popular ornamental plant (Kharrazi et al. 2011; Esmaiel et al. 2013). The greatest multiplication efficiency of the mentioned species was obtained indirectly via callus stage, after enrichment of MS medium with different concentration of BAP (from 0 to 4 mg/L) and NAA (from 0.1 to 1.0 mg/L), while the best regeneration of our *D. carthusianorum* calamine ecotype was achieved by axillary branching on MS medium supplemented with the same type of BAP, but another auxin, that is IAA. Nevertheless, the addition of BAP to medium for Carthusian pink micropropagation brought about vitrified shoots. Kharrazi et al. (2011) reported that the number of malformed shoots in *D. caryophyllus* culture increased on BAP-containing media with the increasing concentration of this cytokinin equivalent. This type of disorder in regenerated plantlets, that affects the production at commercial level and causes difficulties during acclimatization, might be a result of incorrectly chosen concentration of kind of plant growth regulator or their level in the medium or the lack of optimization of other culture conditions (Ivanova and van Staden 2008; Chandra et al. 2010; Kharrazi et al. 2011). Thus, modified MS medium with the addition of 1.0 mg/L BAP supplemented with 0.2 mg/L NAA or IAA should be eliminated from experimental scheme and 1.0 mg/L 2iP with 0.2 mg/L IAA were chosen for clonal propagation of *D. carthusianorum*.

Although in vitro culture allows to a relatively quick production of large amounts of high quality, uniform plant material regardless of the time of year and weather conditions, *ex vitro* microplants acclimatization is still considered a critical step in micropropagation scheme. Thus, it is estimated as the main limitation using this technology on commercial scale (Chandra et al. 2010; Deb and Imchen 2010). During in vitro cultivation, plantlets grow in ambient temperature (25 ± 2 °C) under low light intensity, hence direct transfer to broad spectrum sunlight and *ex vitro* temperature (26–36 °C) might result in their quick wilting and dying (Lavanya et al. 2009; Matysiak and Gabryszewska 2016). Regardless of such environmental factors, the high mortality level of plants being transferred to natural conditions may be a

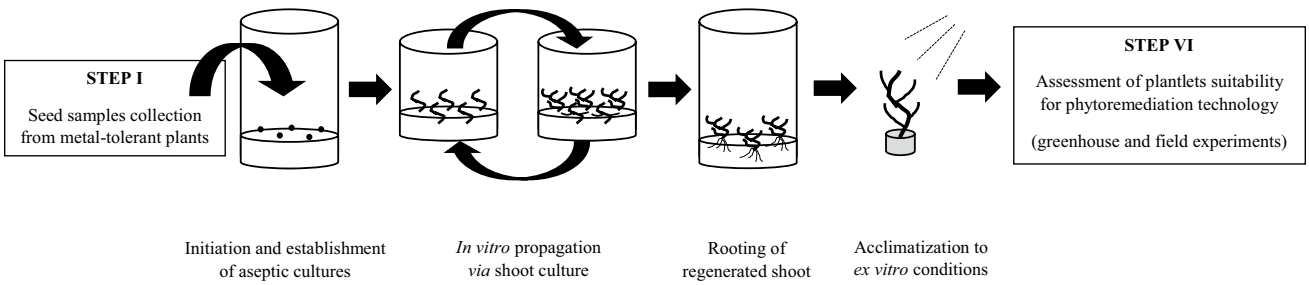

Fig. 5 An abbreviated scheme of successive steps which should be proceeded to obtain plant material ready to be grown in polluted sites

result of sudden exposure to numerous other stress factors at the same time. One of them is the low root system ability to compete with antagonistic microbial soil communities. It is therefore necessary to accustom the plants to such unfavourable conditions by biotization of tissue cultured plantlets with useful microorganisms that promote growth and encourage mutual association (Adriaensen et al. 2003; Senthilkumar et al. 2008; Parray et al. 2015; Quambusch et al. 2016). During our experiments, well-rooted microplants representing calamine ecotype of *D. carthusianorum*, after efficacious acclimatization to greenhouse conditions, were successfully transplanted to contaminated substratum without any additional treatments. We recorded the high survival rate (about 93%) of the specimens being adapted to the natural conditions.

It is now widely accepted that stabilization of wastes disposed after ore exploitation by vegetation cover is far more desirable than physical or chemical methods of remediation (Tordoff et al. 2000; Mendez and Maier 2008; Sheoran et al. 2013; Yang et al. 2016). Successful revegetation is treated as an ecologically justified, permanent, visually attractive and relatively inexpensive solution. Although such an approach is reasonable, metalliferous wastes create very unfavourable conditions for plant development due to the presence of many growth-limiting factors, particularly elevated levels of heavy metals, which can result in deprivation of unsuitable assorted vegetation (Ciarkowska and Hanus-Fajerska 2008). Therefore, the introduction of metal-tolerant plant on chemically degraded areas and stabilization of mine wastes seem to be a guarantee of complete, long-term success. Specimens of *D. carthusianorum* calamine ecotype obtained via micropropagation were able to grow on waste post-flotation material and stabilize it at the same time. Such wastes are characterized by almost complete lack of organic matter, very low nitrogen level, large contents of soluble forms of zinc (115.1 mg kg^{-1}), lead (0.91 mg kg^{-1}) and cadmium (3.12 mg kg^{-1}) and the low water capacity (18.95% g/g) (Muszyńska et al. 2013). Despite these disturbances both in the physical and chemical properties of the mentioned ground, our tested plant ecotype grew and developed properly. Moreover, in the second year of *ex vitro* cultivation on post-flotation wastes, obtained under in vitro conditions plants were more vigorous, had bigger diameter and produced more shoots from root collar than plants obtained by seed sowing. It may ensure not only the suitability of examined plant species to stabilize loose wastes disposed after Zn-Pb ores enrichment, but also the suitability of shoot cultures to provide a large amount of valuable, high quality plant material with the intention to direct use for phytoremediation purpose.

Conclusions

Currently, methods based on metal-tolerant species representing local populations that are well adapted to growth and development in habitats strongly deformed by human activities are being promoted. However, this approach is rarely taken into consideration, even though it can bring additional benefits, which can be gradual reduction of ground toxicity. Then, the requirement of renaturalization and remediation of degraded areas are fulfilled at the same time. As a result of the presented experiment, the conditions of *D. carthusianorum* calamine ecotype culture have been determined in detail. The elaboration of micropropagation protocol allows conducting both basic and applied research which may refer to stress physiology or biochemical and genetic basis of metal tolerance as well as improvement of environmental technologies. With the use of tissue culture techniques it is possible to obtain a great deal of regenerants and thus their preliminary verification for in vivo experiments is feasible in a short time. It was found that micropropagated specimens of calamine *D. carthusianorum* ecotype were able to grow and develop on heavy metals polluted ground. Thus, in vitro propagation should be proposed as a simple, suitable method for efficient production of plant material with potential to stabilize toxic metalliferous wastes. Besides the reduction of the environmental pollution, planting of Carthusian pink calamine ecotype may be a visually attractive solution, and simultaneously relatively inexpensive.

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Author contributions EM developed the concept and performed the experiments, analysis and interpretation of data, as well as drafted the manuscript. EH-F designed experiments, fully participated in data interpretation and in manuscript writing.

Compliance with ethical standards

Conflict of interest The authors hereby declare no conflict of interest.

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